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Award Number: W81XWH-11-1-0767

TITLE: "Rethinking Drug Treatment Approaches in ALS by Targeting ABC Efflux Transporters"

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REPORT DATE: October 2012

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2012	Annual	30 September2011-29September2012

4. TITLE AND SUBTITLE

Rethinking Drug Treatment Approaches in ALS by Targeting ABC Efflux Transporters

5a. CONTRACT NUMBER

5b. GRANT NUMBER W81XWH-11-1-0767

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Thomas Jefferson University
Philadelphia, PA 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Despite multiple therapeutic efforts targeting a variety of underlying pathogenic mechanisms, approaches to cure the mouse the models amyotrophic lateral sclerosis (ALS) have failed. With the exception of Riluzole (the only drug approved by the FDA for treatment of ALS), we have been unsuccessful at translating promising results from pre-clinical mouse trials to effective pharmacotherapies for ALS patients. One of the problems in finding highly efficacious treatments in ALS may derive from the so far underestimated issue of disease-driven pharmacoresistance mediated by the multi-drug resistance (mdr) efflux transporter, P-glycoprotein (P-gp). These are proteins that are present at the blood and spinal cord brain barrier whose function is to protect the brain from xenobiotics including drugs. These proteins actively pump out from the nervous system (CNS) "foreign" substances. We have shown that in ALS, both in patients and in the ALS mice, there is an increased expression and activity of these efflux transporter P-gps and hypothesized that one of the problem in treating ALS derives from a disease-driven acquired pharmacoresistance due to increased P-gps. Riluzole, which only has a modest effect in patients, is a Pg-p substarate. Thus, it is plausible that administration of Riluzole in combination with a P-gp inhibitor could improve its therapeutic outcome. With this proposal we test the hypothesis ***that co-administration of Riluzole with a potent P-gp inhibitor (Elacridar) will enhance Riluzole bioavailability and therefore will improve its therapeutic efficacy the SOD1-G93A ALS mice.***

15. SUBJECT TERMS

Drug resistance, ALS, Therapy, Riluzole, Drug Efflux Transporters

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

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Abstract:

Introduction (Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research):

Despite multiple therapeutic efforts targeting a variety of underlying pathogenic mechanisms, approaches to cure the mouse the models amyotrophic lateral sclerosis (ALS) have failed. Moreover, with the exception of Riluzole (the only drug approved by the FDA for treatment of this disease), we have been unsuccessful at translating promising results from pre-clinical mouse trials to effective pharmacotherapies for ALS patients. Growing evidence indicates that one of the problems in finding highly efficacious treatments in ALS stems from the so far underestimated issue of drug bioavailability and disease-driven pharmacoresistance mediated by the multi-drug resistance (mdr) efflux transporter, P-glycoprotein (P-gp). P-gp is a member of the ATP-binding cassette (ABC) family of proteins that are predominantly present at the blood and spinal cord brain barrier; normally, these proteins maintain cellular homeostasis by actively pumping "foreign" substances—including drugs--from the central nervous system (CNS). P-gp's role in mediating pharmacoresistance is of particular interest due to its broad substrate recognition, which ranges from HIV protease inhibitors to antibiotics. We have previously described an upregulation of P-gp expression and function throughout disease progression in the spinal cord of the SOD1-G93A mouse model of ALS and confirmed an increased expression in post-mortem spinal cord tissue of ALS patients (Boston-Howes et al, 2008; Jablonski et al., 2012). In accordance with these findings, pre-clinical tests in our lab with the potent and specific glutamate uptake enhancer and P-gp substrate nordihydroguaiaretic acid (NDGA) yielded only a transient increase in glutamate uptake in the ALS mice with no effect on survival (Boston-Howes et al., 2008). Interestingly, Riluzole,, which has a modest effect on ALS mouse survival and in patients with the disease, is also a P-gp substrate. Therefore, it is plausible that the modest effect of Riluzole results at least in part from this disease-driven increase in P-gp expression and function that reduces the drug's bioavailability to target tissues in the CNS. Given this reasoning, it's also possible that administration of Riluzole in combination with a P-gp inhibitor could improve the therapeutic outcome of this drug. The experiments in this proposal will test the hypothesis ***that co-administration of Riluzole with the potent and specific P-gp inhibitor , Elacridar, will enhance Riluzole bioavailability and improve its therapeutic efficacy the SOD1-G93A ALS mice.***

Body (describe the research accomplishments associated with each task outlined in the approved Statement of Work):

CHRONIC ELACRIDAR TOXICITY:

STEP 1-ELACRIDAR SAFETY TRIAL IN SOD1-G93A MICE: completed; safe

In order to test the effect of Riluzole while pharmacologically inhibiting P-gp, we proposed that Riluzule will be co-administered to the SOD1-G93A mice with the potent and specific P-gp inhibitor, Elacridar (Sequoia Research Products, England). Given the paucity of data regarding chronic Elacridar

treatment, we first designed experiments to evaluate the toxicity of prolonged Elacridar administration in the SOD1-G93A mouse. **These toxicity studies have been completed.** Elacridar was formulated into custom designed matrix-driven delivery pellets that continuously release the active product in a time-controlled manner (Innovative Research of America). Pellets were subcutaneously implanted in three cohorts of SOD1-G93A mice at symptom onset, and all animals were treated for a total of 20 days. The "low" dose cohort (n = 6) received one 5 mg Elacridar pellet every 10 days (ie, two pellets total, 10 mg of Elacridar released over 20 days), the "high" dose cohort (n = 5) received one pellet containing 100 mg of Elacridar released over 20 days, and the "placebo" cohort received a pellet containing no drug (n = 3). Grip strength, weight, and symptoms were monitored during treatment as measures of disease progression, animals were regularly examined for overt signs of toxicity (ie, bleeding), and liver size and gross morphology were evaluated post mortem as further indications of potential toxicity. We detected no differences in any measures of disease progression, and found that **chronic treatment with either the "low" or the "high" dose of elacridar is not toxic to SOD1-G93A mice**. However, the large size of the "high" dose elacridar pellet (5/16" diameter) caused the skin to split and expose the underlying pellet in several of the treated mice. Since the drug dose and treatment time determine the size of the custom pellet, we overcame these technical complications by formulating lower-dose pellets that are equivalent to the "high" dose of elacridar (50 mg/10 days) for co-administration with Riluzole (see below).

METHODS OF RILUZOLE ADMINISTRATION AND REPLICATION OF PREVIOUSLY PUBLISHED EFFECTS:

STEP 2: OPTIMIZING SINGLE ADMINISTRATION OF RILUZOLE IN SOD1-G93A MICE: completed; RIluzole slows down disease progression as reported.

In addition to evaluating the toxicity of chronic Elacridar administration, we also performed preliminary experiments to establish a suitable and reliable method of Riluzole administration. Since Elacridar toxicity experiments revealed the need for implanting two pellets of the inhibitor at once and subcutaneous space is limited in the mice (average weight of 22 grams), we chose to administer Riluzole orally rather than via subcutaneous pellets as we originally outlined in this grant. Furthermore, oral Riluzole administration is the route employed by Gurney and colleagues (1997), who first reported the significant effects of Riluzole administration on ALS mouse survival. In contrast to Gurney and colleagues, however, we proposed to begin Riluzole administration at symptom onset in an attempt to better translate the effects of this treatment and combined elacridar treatment to the human ALS patient population. **Therefore, we designed and completed experiments to test the effects oral Riluzole administration beginning at symptom onset on disease progression of the SOD1-G93A mice.**

Standard mouse chow nuggets were processed into a powder form in a food processor, and Riluzole (Sigma) dissolved in ethanol was added to processed chow to achieve a final Riluzole dose of 125 mg/kg of chow (Gurney et al., 1997). Riluzole-dosed chow was then re-formulated into nuggets using ice trays, nuggets were unmolded, and food was allowed to harden and dry completely. Control chow was made using the same method but without the addition of Riluzole. SOD1-G93A mice were provided with either control chow (n = 6) or Riluzule chow (n = 6) beginning at symptom onset (approximately 90 days in our lab); food and water was accessible ad lib, and food consumption was

monitored weekly. We found that SOD1-G93A mice given control chow lived 160.83 ± 2.75 days while animals treated with Riluzole survived 175.12 ± 3.77 days, and that **oral administration of Riluzole beginning at symptom onset significantly slowed disease progression in the SOD1-G93A mice [($p < 0.05$) Figure 1].**

MEASURE SPINAL CORD AND PLASMA LEVELS OF RILUZOLE:

STEP 3: DEFINE AND OPTIMIZE METHODS TO MEASURE RILUZOLE LEVELS IN CNS AND BLOOD/MEASURES OF RILUZOLE BIOAVAILABILITY: Methods Optimized- Analysis is progress.

We first optimized a method for the sensitive, reliable, and quantifiable detection of Riluzole in the CNS by Mass Spectrometry. Wild type and P-gp knockout (P-gp$^{-/-}$) mice received an intraperitoneal injection of Riluzole and, after one hour, animals were sacrificed and spinal cords, brains and blood were collected. Protein was extracted from all samples using an ethanol precipitation protocol optimized for Mass spectrometry analysis, and Riluzole concentration was analyzed using a ThermoFisher Orbitrap XL mass spectrometer interfaced with Eksigent nanopumps connected to a 75 µm nanocolumn (New Objective) self-packed with C18 resin. Results from this optimized method demonstrate that (**a) it is possible to detect and quantify Riluzole in CNS samples and (b) when P-gp is not functional (e.g., P-gp$^{-/}$ mice) Riluzole levels are significantly increased in both brain and spinal cord, suggesting that, it is possible to increase Riluzole bioavailability by inhibiting P-gp as we originally hypothesized in this application** (See Figure 2). Since the study is ongoing, samples from SOD1-G93A mice treated with both Riluzole and the P-gp inhibitor, Elacridar, are currently in the collection phase and will be analyzed at the end of the trial.

EVALUATE THE EFFECT OF ELACRIDAR ALONE ON ALS MICE:

STEP 4: CLINICAL STUDY WITH RILUZOLE+ ELACRIDAR: Ongoing

Since these initial experiments completed in our lab determined that that chronic Elacridar aministration is safe in the SOD1-G93A mice**(Step 1),** oral administration of Riluzole starting at disease onset slows disease progression in the SOD1-G93A mice by 15 days **(Step 2),** and, proving the first half of our main hypothesis, eliminating P-gp activity can increase Riluzole bioavailability in the CNS **(Step 3)**, **we have begun our study to test the remaining hypothesis that *co-administration of Riluzole with the potent and specific P-gp inhibitor , Elacridar, will improve Riluzole's therapeutic efficacy in the SOD1-G93A ALS model mice.*** As outlined in the original proposal, the trial requires a large number of mice to be assigned to the following experimental groups:

For disease progression and survival curve

Elacridar alone = 15 mice

Riluzole alone = 15 mice

Elacridar + Riluzole= 15 mice

For histological and pathological analysis

5 additional mice/experimental group described above (15 mice, total).

We have now 3 mice/group analyzed; preliminary data show an encouraging prolongation of Riluzole effect in the animals (n=3) treated with Elacridar.

Key Research Accomplishments (Bulleted list of key research accomplishments emanating from this research):

1. We have proved our hypothesis that inhibiting P-gp function (albeit genetically) increases the bioavailability of Riluzole in the CNS, thus supporting our rationale for the ongoing co-treatment of SOD1-G93A mice with Riluzole + Elacridar, as outlined in this proposal.

2. We have completed the safety trial of Elacridar chronic administration: chronic Elacridar treatment is safe in the SOD1-G93A mice.

3. We have demonstrated that Riluzole administration beginning at symptom onset is modestly yet significantly effective in slowing disease progression of the SOD1-G93A mouse model of ALS, confirming previous reports of Riluzole efficacy in the mice and recapitulating Riluzole efficacy in the ALS patients.

4. The mandatory experiments from Steps 1 to 3 have been completed successfully, thus setting the stage for the core pre-clinical trial of co-treatment with Riluzole + Elacridar in the ALS mice.

Reportable Outcomes (list):

A manuscript is in preparation reporting positive results from Step 1 to 3 and awaiting final analysis of step 4.

Conclusion (Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report):

As summarized above in “Key Research Accomplishments” the experiments performed in year 1 of the project have successfully proved our hypothesis that P-gp inhibition improves Riluzole bioavailability to the CNS. Technically, the experiments performed in year 1 have also strengthened our rationale for performing the pre-clinical trial at the core of the proposal which will test whether P-gp inhibition enhances the therapeutic efficacy of Rilzule in the SOD1-G93A mice. We feel that the experiments are on time and that we are on target with our original goals. There have not been major modification and/or deviations from the original proposal except for altering the route of Riluzole administration from subcutaneous pellet implantation to oral administration in the mouse foodas described above in STEP 2.

References:

Boston-Howes W, Williams EO, Bogush A, Scolere M, Pasinelli P, Trotti D. (2008) Nordihydroguaiaretic acid increases glutamate uptake in vitro and in vivo: therapeutic implications for amyotrophic lateral sclerosis. Exp Neurol, Sep; 213(1):229-37.

Jablonski MR, Jacob DA, Campos C, Miller DS, Maragakis NJ, Pasinelli P, Trotti D. (2012) Selective increase of two ABC drug efflux transporters at the blood-spinal cord barrier suggests induced pharmacoresistance in ALS. Neurobiol Dis, Aug;47(2): 194-200.

Appendices:

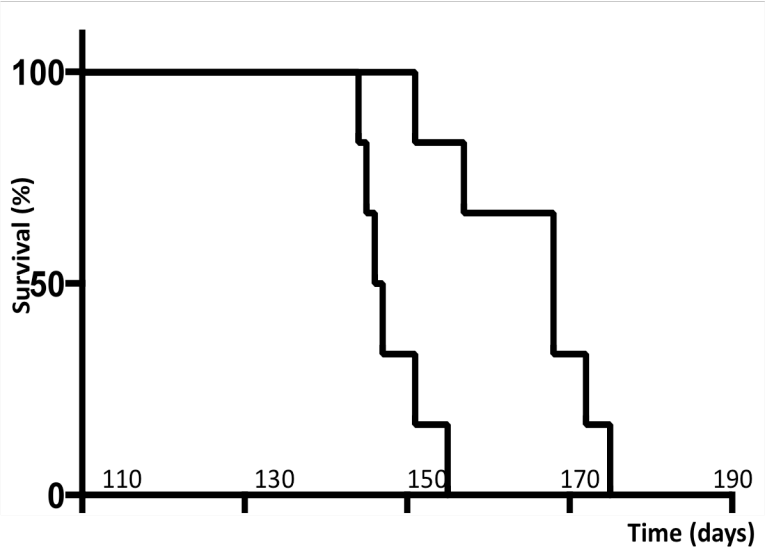

FIGURE 1: ADMINISTRATION OF RILUZOLE AT DISEASE ONSET SIGNIFICANTLY SLOWS DOWN DISEASE PROGRESSION IN THE SOD1-G93A MICE. Kaplan-Meyer survival curves of untreated SOD1-G93A mice (left curve) and Riluzole-treated SOD1-G93A mice (right curve).

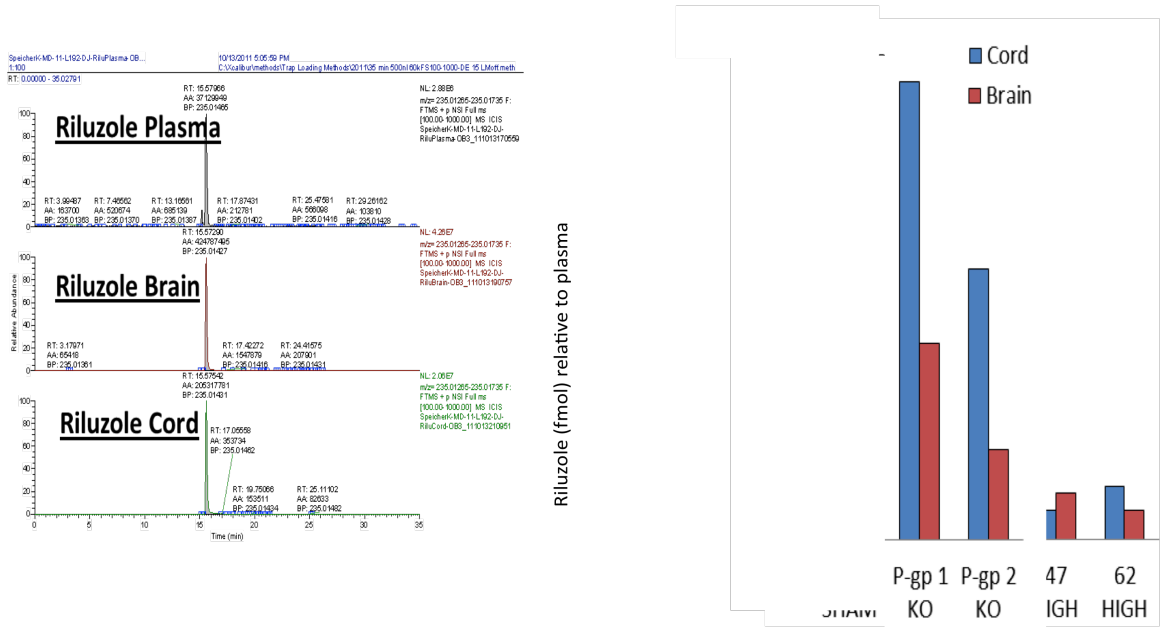

FIGURE 2: P-gp INHIBITION INCREASES RILUZOLE CONCENTRATION IN SPINAL CORD AMD BRAIN. As determined by LC/MS, in mice knock-out for P-gp. Riluzole levels increase in both brain (red bars) and spinal cord (blue bars) compared to control mice. Left graphs are representative of LC/MS analysis indicating Riluzole picks.

Supporting Data: